



Review

## Deoxyribonucleic acids as unique markers in molecular detection

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Received 24 March 1997; received in revised form 3 April 1997; accepted 4 April 1997

Deoxyribonucleic acids (DNAs) are unarguably one of the most easily manipulated biological materials. This can be proved by the remarkable developments in molecular cloning techniques and recombinant DNA technology over the last few decades. This characteristic, derived from a number of unique properties of DNA, can also allow DNAs to be used as versatile markers for detection of various targets. In the first section of this article, key properties of DNA that make it a particularly attractive marker in molecular detection will be described for readers who are not familiar with basic characteristics and manipulation of DNA. Then, we will provide examples of how DNA markers can actually be used effectively in such detection schemes with emphasis on immuno-PCR technology.

### 1. Unique properties of DNA

Several unique properties of DNA allow it to serve as a versatile marker in various detection schemes. There are also many techniques available for manipulation and analysis of DNA, some of which are of key importance when DNA is used as a marker. These characteristics and techniques are briefly discussed below.

DNA is one of the most monotonous biological molecules, because it consists of only four basic elements, called bases. These bases are linked by a backbone consisting of alternating deoxyribose sugar and phosphate moieties, called a phosphodiester backbone. The order of bases along a DNA strand is called the sequence which determines the properties of the DNA.

DNA is very stable both chemically and physically, particularly in the absence of divalent cations that are needed for activities of many DNA-degrading enzymes, called nucleases. The simple structural nature and stability allow DNA to be manipulated easily by various in vitro techniques.

Single-stranded DNA molecules recognize their complementary sequences with great specificity to form double-stranded DNA. This process, called hybridization, is governed by specific recognition of pairs of bases through hydrogen bonding. The process occurs spontaneously for relatively short DNA molecules. The specificity of hybridization between a pair of complementary DNA sequences is so high that, under appropriate conditions, molecules with perfect double-strand formation can effectively be discriminated from those containing imperfect base recognition by the difference in stability of the duplexes.

DNA can be easily modified by both chemical and enzymatic means. For example, tags, such as fluorescent dyes and enzymes, can be attached chemically to a DNA strand which is then detectable through the attached tag. A variety of DNA modifying enzymes are available, many of which act on DNA in a sequence-specific manner. For instance, a DNA strand can be cleaved specifically at particular sequences by enzymes, called restriction endonucleases; a short DNA strand can be extended along a longer DNA molecule by sequence-specific DNA polymerase; and two or more DNA strands can be joined covalently by DNA ligase.

Relatively short single-stranded DNA molecules, up to about 100 bases long, can easily be generated chemically by using an automated DNA synthesizer. In order to make longer DNA molecules, sets of double-stranded DNA are prepared by hybridization of pairs

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of chemically synthesized, complementary single-stranded DNA strands, and the resulting double-stranded DNA molecules are joined by ligation. By this method, DNA of any sequence and length can, in principle, be generated.

DNA can be amplified biologically or enzymatically. Particular forms of DNA molecules, such as bacterial plasmids and bacteriophage genomes, can be amplified biologically by simply growing them in host organisms, most typically the bacterium *Escherichia coli*, which carry the DNA. Various modifications have been made on such DNA molecules to allow them to carry foreign DNA sequences. There are also a few in vitro enzymatic DNA amplification methods that are frequently used in the laboratory, and many more are being developed. Currently, the most useful in vitro DNA amplification system is the polymerase chain reaction (PCR), which can selectively amplify a particular DNA segment, defined by a set of short DNA molecules called primers, in complex mixtures of DNA fragments.

DNA can be fractionated by size using gel electrophoresis. The size resolution of gel electrophoresis for DNA is considerably higher than that used for other biological materials. For example, one-base resolution can routinely be obtained for single-stranded DNA of up to at least 1000 bases long. The maximum size limit of fractionation by gel electrophoresis with reasonable resolution exceeds several million base pairs, and thus it is in a size range which includes many bacterial genomes.

## 2. Immuno-PCR technology

Unique characteristics of DNA, along with analytical methods available for detection and manipulation of DNA, should allow DNAs to serve as powerful, versatile markers in various detection systems. Our effort to develop sensitive detection methods for biological targets using DNA as a marker was initiated by coupling PCR to antibody-based antigen detection (immunoassay) systems. Immunoassays are one of the most versatile detection methods for a variety of biological and non-biological targets and they are widely used in molecular and cellular analyses. Almost all current immunoassays involve particular markers for signal generation and detection, which include radioisotopes for radioimmunoassays and color-generating enzymes for enzyme-linked immunosorbent assays (ELISA). Such markers are generally attached to targets (antigens) via antibodies. By using a similar configuration [1-6], shown schematically in Fig. 1, arbitrary, exogenous DNA could be attached specifically to antibody and used as a marker for detection of antigen. The DNA molecule, attached to target antigen through antibody, allows the amplification of its partic-

ular segment by PCR or other in vitro DNA amplification systems to generate detection signals. The amplified DNA segments (PCR products) are analyzed to see if the antigen is present in the assay sample.

In the first immuno-PCR format [1], a recombinant chimeric protein between a biotin-binding protein, streptavidin, and an immunoglobulin G (antibody)-binding protein, protein A [7], was used to attach marker DNA, into which a biotin moiety had been incorporated, to antigen-antibody complexes immobilized on a solid support. PCR amplification of a segment of marker DNA attached to antigen-antibody complexes, followed by analysis of PCR products, allowed extremely sensitive, specific detection of target antigen. In a model system, in which standard gel electrophoresis was used to analyze PCR products, immuno-PCR offered several orders of magnitude higher sensitivity than conventional ELISA, demonstrating the distinct power of DNA markers in immunoassays.

Since the first demonstration of immuno-PCR [1], several modified formats have been developed. One modification involves a mono-specific multivalent binding protein, such as streptavidin or avidin, which is used to attach biotinylated marker DNA to biotinylated antibodies bound to target antigens [8-10]. Another modified format uses chemical, covalent conjugates between antibody and marker DNA without the use of a linker molecule [11,12]. A similar modification involves a chemical conjugate between streptavidin and marker DNA [13], which can be targeted to biotinylated antibody-antigen complexes.

Recently, the multiplexing capability of immuno-PCR was demonstrated experimentally by using antibody-marker DNA chemical conjugates, each of which is directed to a different antigen [11]. In this scheme, different-sized PCR products are generated, each of which is specific to a particular antigen. This multiplexing capability, which utilizes the specificity of each primer-marker DNA pair in PCR and the ability to fractionate different-sized DNA segments generated by PCR, allows simultaneous detection of many different antigens in single assays.

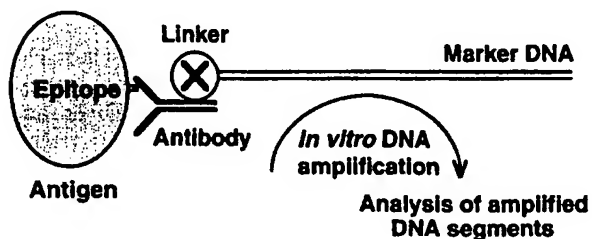


Fig. 1. Concept of DNA marker used in immunoassays. Modified from Sano [3].

All of these and other formats have not only provided this technology with greater versatility but have also shown great promise for immuno-PCR to become a standard method in a variety of molecular and cellular analyses, including clinical diagnostics [4,5,14,15].

### 3. Problems and challenges

The immuno-PCR technology offers among the highest sensitivities currently achievable in immunoassays, derived primarily from the enormous amplification capability of PCR. A potentially very serious problem, inherited in sensitive detection methods including immuno-PCR, is background. In immuno-PCR, even minute amounts of non-specific binding or adsorption of marker DNA to other components or solid supports used in the assays could generate significant background signals, thus reducing signal-to-noise ratios. In many sensitive detection systems involving powerful signal amplification techniques, the signal strength itself is often not the concern; instead, signal-to-noise ratios are, in many cases, the primary factor to determine the specificity of the assay system. Use of appropriate blocking agents to repress non-specific binding and adsorption is critical. In immuno-PCR, random DNA mixtures, such as sheared sperm DNA, repress background levels considerably, with no appreciable effect on specific signal generation by PCR. Development of solid supports which minimize non-specific binding is certainly helpful. Many new materials, which have not been used previously with biological materials, are being developed and tested to see if they could offer enhanced performance when used as solid supports in biological detection schemes. Molecular engineering of assay components should also help to reduce background levels. For example, genetic engineering of an antibody to enhance the affinity for its antigen should allow the use of more stringent washing conditions that facilitate the removal of weakly bound complexes without disturbing specific antigen-antibody interactions.

Similarly, contamination of marker DNA into other assay reagents or assay environments causes very serious problems, because positive signals will be generated independent of the samples. One particularly attractive aspect of immuno-PCR is that marker DNA used is purely arbitrary, so that it can be changed frequently, as needed, to avoid deterioration of signals or signal-to-noise ratios. This is a distinct advantage over other detection methods in which sample-derived nucleic acid targets are directly amplified by PCR or other *in vitro* amplification systems.

Another potential problem of immuno-PCR is that it may provide rather low accuracy in quantitation of a target; this problem arises because PCR is not generally

very quantitative. Many powerful signal amplification systems have the same drawback. This problem becomes even more serious when samples are complex mixtures or contain only trace amounts of targets.

One potential way to circumvent this problem is to develop detection systems without signal amplification, although this is generally very difficult, particularly when high detection sensitivity is needed. In principle, such systems could be developed if large numbers of labels, sufficient for detection without amplification, could be attached specifically to each target molecule. In such schemes, DNA could be used as a signal carrier, instead of a marker, to attach many labels to single targets. For example, it would be very attractive if arbitrary DNA could be used to carry large but known numbers of labels and such label-containing DNA carriers could be directed specifically to targets. If sufficiently large numbers of labels could be attached to each target via single DNA molecules, no signal amplification should be needed for detection, and accuracy in quantitation of targets could be increased accordingly. Because the length and sequence of DNA can be controlled at will, such DNA carriers containing many label molecules should be designed and prepared relatively easily.

### 4. Perspectives

The uniqueness and power of DNA markers in molecular detection have been described by using immuno-PCR as an example of how DNA markers can actually be used in practice. Here, a key consideration is that the application of DNA markers is not limited to immunoassays; instead, the sensitivity and specificity of many existing detection systems could, undoubtedly, be enhanced and improved by incorporating DNA markers into such systems. Coupling to many emerging technologies for manipulation and analysis of DNA should also expand the potential of DNA markers to be applied to the detection of a wider range of biological and non-biological targets.

### Acknowledgements

The work described in this article was supported by grants from the National Cancer Institute (CA39782) and the National Institute of Standards and Technology, US Department of Commerce (70NANB4H1509).

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